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INJECTIONS OF CALCIUM IONS INTO SPINAL MOTONEURONES

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SUMMARY

1. In cats under Dial anaesthesia, Ca^{2+} was injected inside lumbosacral motoneurones, by passing currents between $CaCl_2$- and KCl-containing barrels of compound micropipettes.

2. There was a reduction in excitability and a fall in membrane resistance, both rapid in onset and quickly reversible.

3. The minimum effective injection current was $\approx$ 10 nA, and the effect reached a maximum with currents of $\approx$ 30 nA. The mean slope of resistance change against injection current was $-1{\cdot}7$ %/nA (S.E. 0·35).

4. The most common change in membrane potential was a hyperpolarization; but in nearly half the cases, there was no clear change or a small depolarization. A reversal level for the effect of Ca^{2+} could be measured in five cells: on the average, it was 10 mV more negative than the resting potential.

5. Observations on i.p.s.p.s showed that Ca^{2+} probably does not alter g_{Cl}: it was concluded that the fall in membrane resistance caused by intracellular Ca^{2+} is mainly due to an increase in g_K.

6. These results confirm previous suggestions that a steep transmembrane gradient of Ca^{2+} is essential for the maintenance of a low membrane conductivity, and that a rise in internal free Ca^{2+} – whether due to influx or release from internal stores – may play an important role in regulating neuronal activity.

INTRODUCTION

This study originated from the observation that 2,4-dinitrophenol (DNP) reversibly blocks the excitability of cortical neurones by inducing a rise in K^+ conductance (g_K) (Godfraind, Krnjević & Pumain, 1970; Godfraind, Kawamura, Krnjević & Pumain, 1971). It was suggested that this change in membrane permeability may be caused by an increase in cytoplasmic free Ca^{2+}, resulting from the slowing down of Ca^{2+} uptake by

mitochondria. This suggestion was prompted by the well-established fact that the mitochondria of various tissues absorb Ca^{2+} by an active process, which is readily blocked by DNP (Vasington & Murphy, 1962; Rossi & Lehninger, 1963; Chance, 1965; Lehninger, 1970; Carafoli & Rossi, 1971). The importance of mitochondria for the intracellular storage of Ca^{2+} in the squid axon has been emphasized recently by Baker, Hodgkin & Ridgway (1971).

Although there is growing evidence that a rise in free Ca^{2+} inside red blood cells leads to an increased membrane permeability to K^+ (Whittam, 1968; Lew, 1970; Romero & Whittam, 1971), the only information pertinent to neurones is a brief report by Meech & Strumwasser (1970) that injections of Ca^{2+} into *Aplysia* neurones cause a sharp rise in g_K. The present experiments were therefore undertaken in an attempt to study the effects of injections of Ca^{2+} on some central neurones in mammals. Preliminary reports of the results have already appeared (Feltz, Krnjević & Lisiewicz, 1972*a*, *b*).

METHODS

Results were obtained from fourteen cats, anaesthetized with Dial compound (diallyl barbituric acid and urethane) in doses of 0·7 ml/kg I.P. In most cases, the animals were allowed to breathe spontaneously, but in five experiments, to reduce movements to a minimum, they were given succinylcholine chloride (Anectine, Burroughs Wellcome) by slow I.V. infusion, and, after a bilateral pneumothorax, were maintained on artificial respiration. The arterial pressure was monitored throughout, and was kept over 60 torr by infusion of saline or, if necessary, noradrenaline.

Preparation

The lumbo-sacral spinal cord was exposed by laminectomy; the cord and dura were cut at the first lumbar segment, and the last lumbar and first sacral dorsal and ventral roots were cut and set up for stimulation. At the end of this preparation, the spinal cord was covered with a deep layer of medicinal liquid paraffin held in a pool over the back by the raised skin flaps. The temperature of the cat, measured both with a subscapular thermometer and at the surface of the cord, in the paraffin pool, was kept above 35° C by infra-red lamps.

Intracellular recording and injections of Ca^{2+}

In the initial experiments, double-barrelled micropipettes were used, one barrel containing 3 M-KCl and the other 0·2 M-$CaCl_2$ (according to previous experience (Kato & Somjen, 1969), this concentration is more satisfactory for microiontophoresis than 0·5 or 1 M). However, it soon became evident that substantial injections of Ca^{2+} were necessary and that any effect produced might not greatly outlast the period of injection. In an attempt to overcome the problem posed by the gross changes in membrane potential induced by the iontophoretic currents we modified the technique as follows.

Three-barrelled micropipettes were made from Kimax glass tubing; after the barrels were fixed together, they were drawn out in two stages, first by hand and then with

a de Fonbrune microforge, following the procedure outlined by Burke & ten Bruggencate (1971). Apart from having three barrels, the electrodes differed from those described by these authors in having a shorter final segment (seldom longer than 10 μm) and a tip with a less regular 'hypodermic' shape. The tip diameter of useful electrodes did not exceed 2–3 μm. The electrodes were filled first with water, which was replaced with 3 M-KCl for two of the barrels, and 0·2 M-$CaCl_2$ for the third.

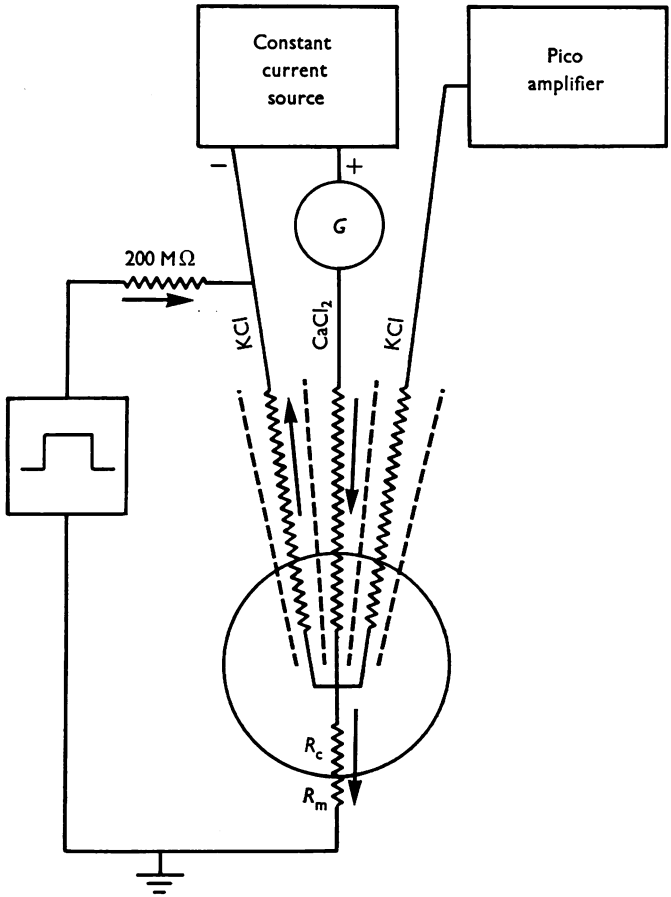

Fig. 1. Diagrammatic representation of three-barrelled micropipette with tip inside a cell, and connexions to Picometric input stage, a unit providing rectangular waves for tests of resistance and excitability, and a source of constant current for iontophoresis; G is galvanometer, R_c coupling resistance at tip, and R_m cell membrane resistance.

The channel resistances (with the tip in saline) were of course much higher than in the experiments of Burke & ten Bruggencate (1971), being mostly in the range of 15–30 MΩ.

The recording (KCl) barrel was connected to the input of a Picometric amplifier (Instrumentation Lab, Boston) via saline and a Ag-AgCl junction. This amplifier

has a high input impedance (> 10^{10} Ω) and provision for compensating the input capacitance to ground. The frequency response was monitored by applying a triangular wave form to the input through a 1 pF condenser (Lettvin, Howland & Gesteland, 1958).

The other two barrels were connected through platinum wires to a floating source of adjustable constant current. Thus, a known current (monitored to the nearest 1 nA on a series galvanometer) could be made to flow between the $CaCl_2$ and one of the KCl barrels. This KCl barrel was also connected through a 200 MΩ resistor with

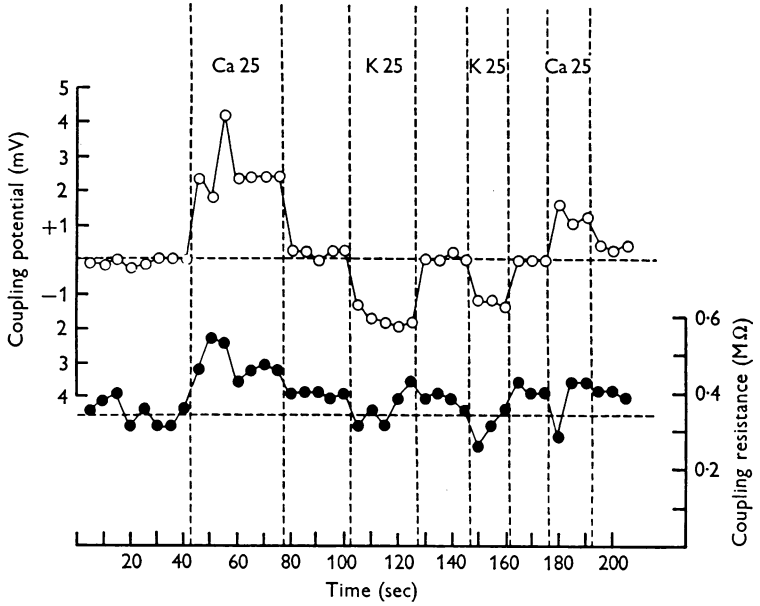

Fig. 2. Control run showing changes in extracellular potential (open circles) and resistance (filled circles) recorded by three-barrelled micropipette during release of Ca^{2+} or K^+. with currents of 25 nA.

a rectangular wave stimulator (otherwise linked to ground) which provided intracellular pulses for tests of membrane resistance and excitability. This system is illustrated diagrammatically in Fig. 1. Although iontophoretic current to ground was greatly reduced, nevertheless, even with a low KCl-barrel resistance (10 MΩ), 5% of the total current would be shunted via the 200 MΩ resistor, the stimulator, ground and finally through the membrane. Thus, the Ca^{2+}-releasing current also had a direct depolarizing effect, which varied from almost negligible to very substantial, depending on the KCl-barrel resistance.

Artifacts of the technique

Very few electrodes permitted the release of more than very limited amounts of Ca^{2+}. As a rule, we applied iontophoretic currents of 10–30 nA, for periods of up to 1 min; even with such moderate currents, the barrel resistance often increased rapidly and repeated injections soon led to an effective block of the electrode.

The release of Ca^{2+} was frequently associated with a progressive rise in tip coupling resistance, as well as a positive shift in potential; whereas currents flowing in the

reverse direction (from KCl to $CaCl_2$) had opposite effects: the tip potential became more negative and the resistance tended to fall. These changes were usually quite evident during extracellular recording, as can be seen in Fig. 2 (cf. also Figs 7*B*, 8*B*).

We can thus distinguish between two kinds of artifacts. One was caused by the shunting of a small fraction of iontophoretic current through ground, as shown in Fig. 1: this tended to depolarize the membrane and increase its excitability, independently of the direction of the iontophoretic current (whether releasing K^+ or Ca^{2+}). The second was a slower, usually reversible increase in tip positivity and resistance, seen only when Ca^{2+} was released.

These artifacts varied greatly in different experiments. Some electrodes were largely free of them; they provided the bulk of useful results, especially with regard to estimates of changes in membrane potential. Although extracellular control runs were always performed, clearly they could be of no more than limited value when the coupling resistance substantially exceeded the probable cell input resistance.

RESULTS

Useful observations were made on sixty-two neurones, mainly motoneurones identified by antidromic invasion from ventral roots.

Whenever recording conditions were sufficiently stable, and reasonably steady iontophoretic currents could be applied through the micropipette without excessive coupling artifacts, tests were made of the effects produced by internal injections of Ca^{2+}, paying particular attention to changes in excitability, resistance and potential.

Changes in excitability

Spikes could usually be evoked by orthodromic stimulation, antidromic invasion or direct intracellular stimulation. Occasionally, the electrode recorded from inside some other kind of cell, identified as a neurone by spontaneous discharges, or spikes evoked by orthodromic or direct stimulation.

Most motoneurones were easily identified by antidromic invasion. On some occasions, however, the electrodes penetrated cells having particularly large and stable resting potentials (≈ -80 mV), though no sizable response could be evoked by stimulating the ventral roots, but a typical, short-latency full-size antidromic spike could be obtained if the membrane was first depolarized (+5–10 mV) by an intracellular injection of current. It is not clear whether this failure of invasion is connected with a possible leakage of $CaCl_2$ and KCl from the electrode, or whether it is a feature of some normal quiescent cells.

Spikes of all types could be depressed by injections of Ca^{2+}. For example, antidromic spikes would show partial or total decomposition, as in the examples of Fig. 3. The second unit in this Figure (*E–H*) was thus reduced to a smaller (*A* or *IS*) component, but a complete spike reappeared when a depolarizing pulse was superimposed (*H*).

Changes in excitability could be demonstrated more directly by eliciting

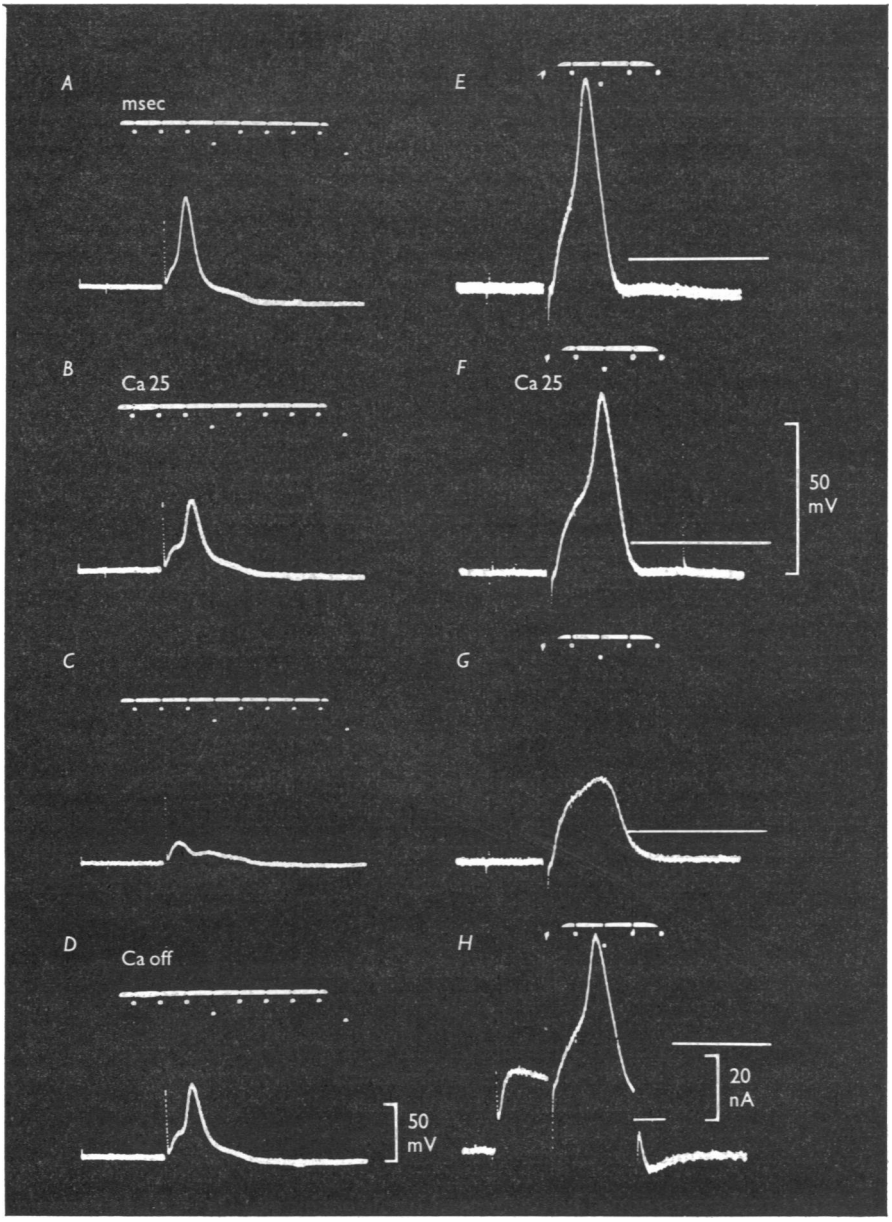

Fig. 3. Two examples of antidromic spikes partly blocked by intracellular injections of Ca^{2+}. In left-hand series, *B* and *C* were recorded 15 and 25 sec after start of injection by current of 25 nA, *D* was 15 sec after its end. In right-hand series, *F*, *G* and *H* were recorded 10, 15 and 20 sec after start of injection; but in *H*, invasion was restored by a depolarizing current pulse, monitored on second trace at right.

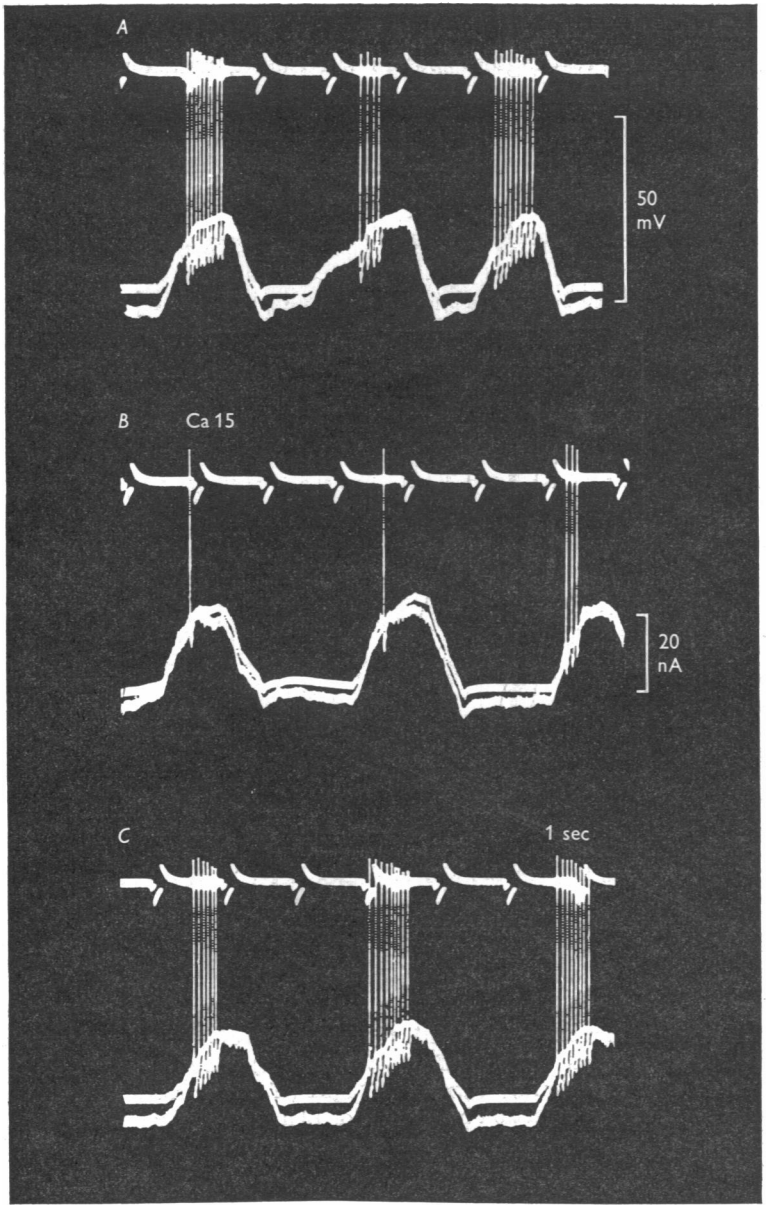

Fig. 4. Excitation by intracellular depolarizing current (monitored on second trace), and depression of firing during injection of Ca^{2+} by current of 15 nA (*B*).

spikes with intracellular injections of depolarizing currents. Such currents were sometimes varied by hand (relatively slowly), as in Fig. 4; more often, 10–20 msec rectangular pulses were used, which were accurately repeatable and could be varied within a range of ± 40–70 nA. Under stable conditions, a sufficient number of responses could be evoked to draw up strength–latency curves (cf. Araki & Otani, 1955; Frank & Fuortes, 1956). For example, the open circles and squares of Fig. 5 plot the latencies of

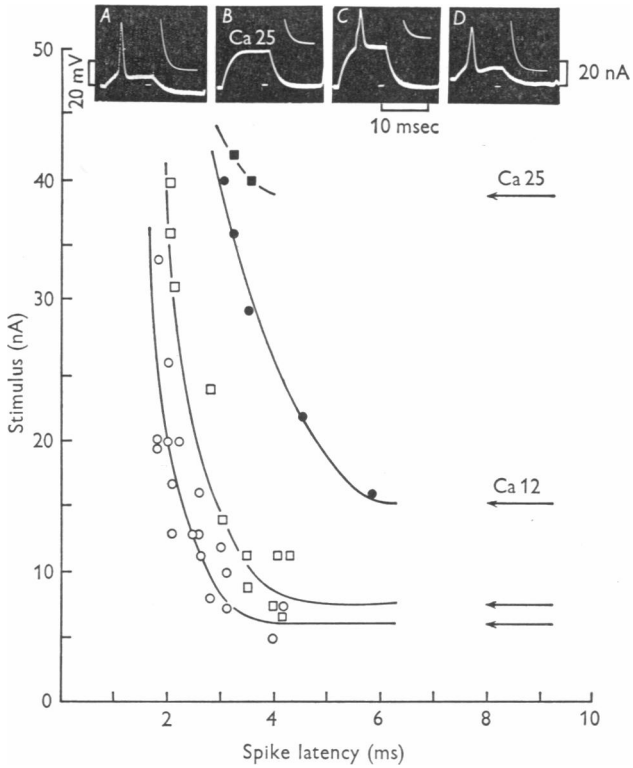

Fig. 5. Strength–latency curves drawn by eye through points obtained by evoking spikes in motoneurone with 10 msec pulses of depolarizing current. Open circles: initial control (cf. inset traces *A*); filled squares: during 15 sec of Ca^{2+} injection by current of 25 nA (cf. inset traces *B*, *C*); filled circles: during Ca^{2+} injection by current of 12 nA; open squares: 30–60 sec after end of Ca^{2+} injections (cf. inset trace *D*; note current pulse is monitored on second oscilloscope trace).

spikes evoked in a motoneurone by 10 msec depolarizing pulses of different intensities, before and after an intracellular injection of Ca^{2+} (cf. inset traces *A* and *D*, where the current pulse is monitored on the second oscilloscope trace). Initially, Ca^{2+} was injected by an iontophoretic current of 25 nA, and during this period, responses could be evoked only with the

strongest pulses available (cf. inset traces *B* and *C*). After some 20 sec, the resistance of the iontophoretic barrels rose sharply, so that the Ca^{2+}-releasing current fell by a half. The data marked by black points were recorded during the following minute. The horizontal arrows in the Figure indicate the approximate level of the rheobase.

Systematic tests of this kind were performed on seven cells. The initial rheobase currents ranged from 6 to 40 nA, with a mean of 19·1 nA. They were thus somewhat higher than those observed by Frank & Fuortes (1956), whose mean was 7·0 nA. Although this difference might suggest some intracellular leakage of Ca^{2+}, a more likely explanation is that larger cells were sampled by our multibarrelled electrodes.

The changes in rheobase were quite variable, but there was a tendency towards a larger relative increase if the initial rheobase current was low (as in Fig. 4). When Ca^{2+} was injected by a current of 25 nA, the increase in rheobase varied between a just significant value of 12 % and a maximum > 500 % (mean of six observations + 151 %).

Although an 8 nA injection of Ca^{2+} caused a 20 % increase in rheobase on one occasion, definite changes were seen consistently only with iontophoretic currents > 12 nA (cf. Fig. 2 in Feltz *et al.* 1972*b*).

These changes in excitability were remarkably quick in onset and rapidly reversible; they reached or approached a maximum within 10–30 sec of the start of the injections, and when the injections were stopped, they largely disappeared within 10–60 sec. Some incomplete recoveries may have been due to deterioration of the recording conditions, masked by artifactual voltage shifts (see below).

Changes in membrane resistance

In view of the consistent tendency towards a depression of excitability, it was of interest to know whether injections of Ca^{2+} were associated with any significant alteration in membrane resistance.

The first method of testing resistance utilized steady currents, or more often, brief (10–20 msec) rectangular pulses. The electrode coupling resistance was balanced out with a bridge circuit when the electrode was in an extracellular position (cf. Araki & Otani, 1955; Frank & Fuortes, 1956; Godfraind *et al.* 1971). Voltage–current lines were obtained by plotting the maximum potential change developed across the membrane (cf. Figs. 5 and 6) against the intensity of applied current; examples of such lines are illustrated in Figs. 7, 8 and 11–13. Controls were performed immediately after moving the electrode out of the cell (Figs. 6*H*, *I*; 7*B*; 11). If necessary, a suitable correction was made when calculating the cell input resistance from the slopes of the voltage–current lines. Not infrequently, a line could be fitted to the data by eye without difficulty; but if the points were relatively scattered, regression lines were obtained by the method of least squares, either a single line for all values of current, if a linear relation was apparent throughout, or separate lines for positive and negative currents.

Fig. 6 illustrates the changes in resistance recorded inside a motoneurone during an injection of Ca^{2+}. Since the traces were obtained at intervals of 5 sec, it is clear that the fall in resistance and later its recovery (after the end of the injection) were both very rapid. These traces also indicate a marked reduction in membrane time constant; but the initial portion of the recorded voltage pulse was too distorted by capacitative artifacts to

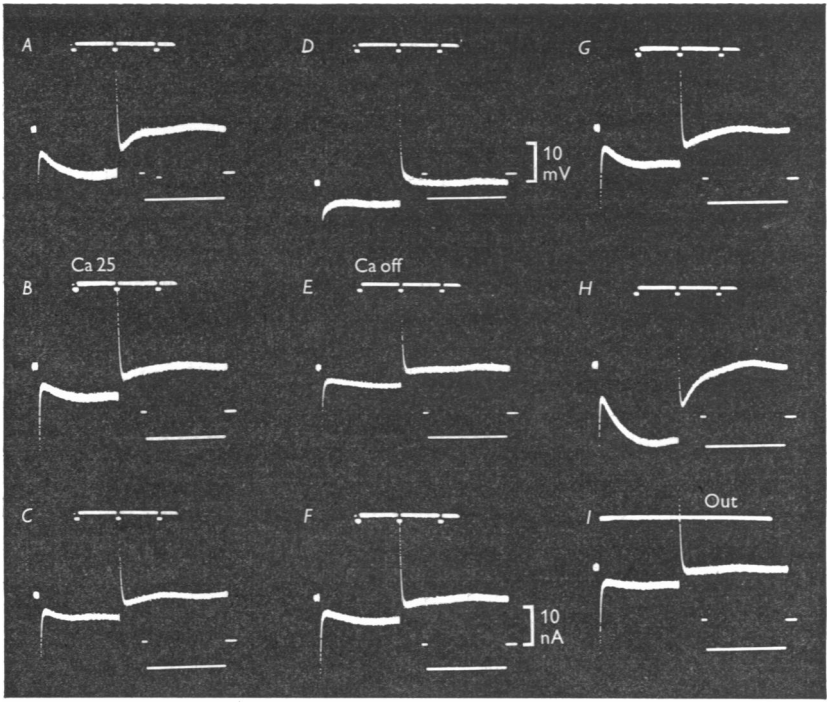

Fig. 6. Voltage changes developed inside cell by application of hyperpolarizing current in rectangular pulses, monitored at right in each frame. Traces *B*, *C*, *D* were recorded at intervals of 5 sec after start of intracellular injection of Ca^{2+} (by current of 25 nA) and *E*, *F*, *G* at 5 sec intervals after end of injection (which lasted 60 sec). *H* was recorded another 60 sec after *G*, and *I* on withdrawing electrode from cell (but after readjusting vertical position). Downward deflexions are in negative direction and time marks indicate 10 msec.

allow accurate measurement of changes in resistance by this parameter (cf. Araki & Otani, 1955; Frank & Fuortes, 1956; Lux & Pollen, 1966; Burke & ten Bruggencate, 1971).

Several examples of voltage–current lines obtained by the application of a series of pulses are illustrated in the two sets of graphs of Fig. 7, in which the black circles represent data recorded during injections of Ca^{2+}. In Fig. 7*A*, the filled squares show the resistance still at a reduced level

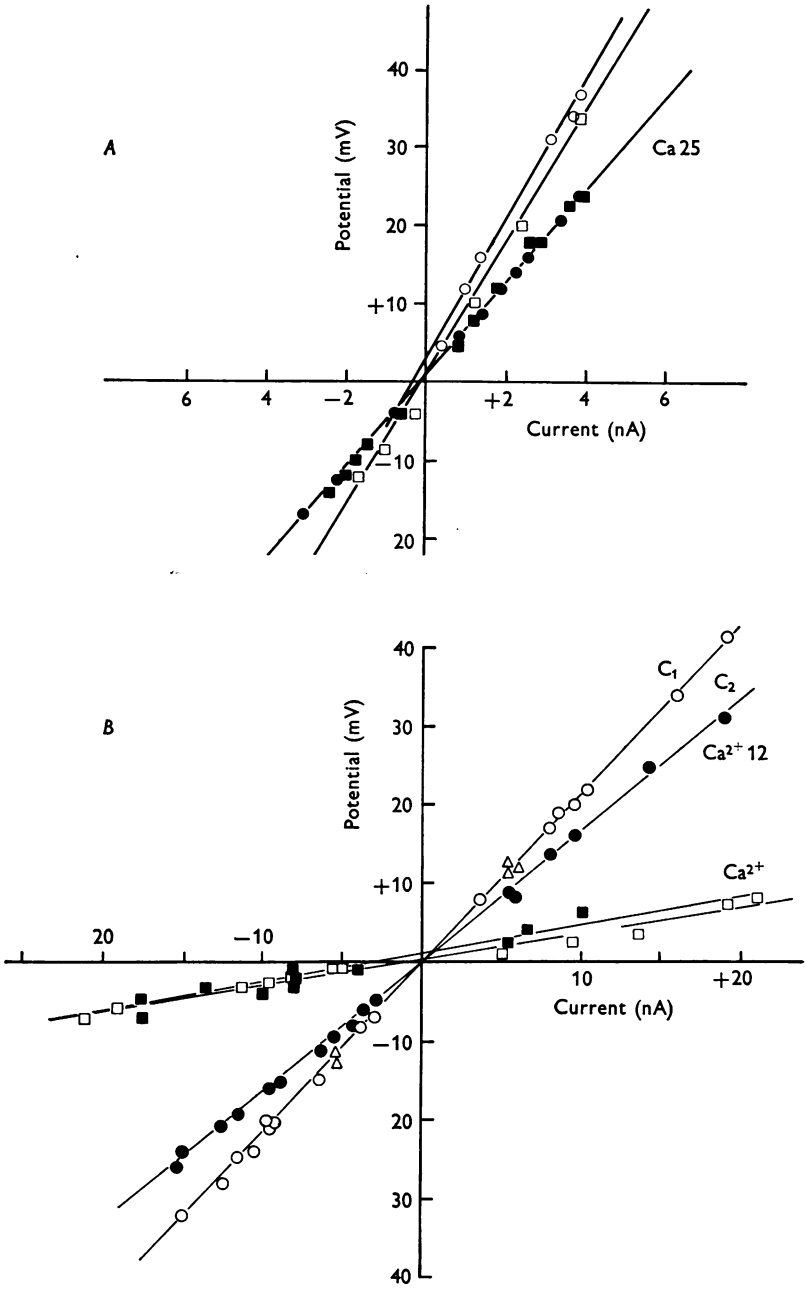

Fig. 7. Voltage–current lines obtained with 10–20 msec pulses from two neurones. *A*, open circles: initial control; filled circles: during injection of Ca^{2+}; filled squares: 10–30 sec after end of injection; open squares: 1 min later. *B*, open circles: initial control; filled circles: during injection of Ca^{2+}; triangles: 10–20 sec after end of injection. Open and filled squares are controls outside cell, before and during Ca^{2+} release. All injections of Ca^{2+} lasted about 1 min.

10–30 sec after the end of the injection, but the further control values recorded 1 minute later indicate a return to the original level (cf. also the triangles in Fig. 7*B*).

In comparable tests on other cells (Figs. 8, 11–13), the voltage–current points did not fall on a straight line. The resistance evidently tended to

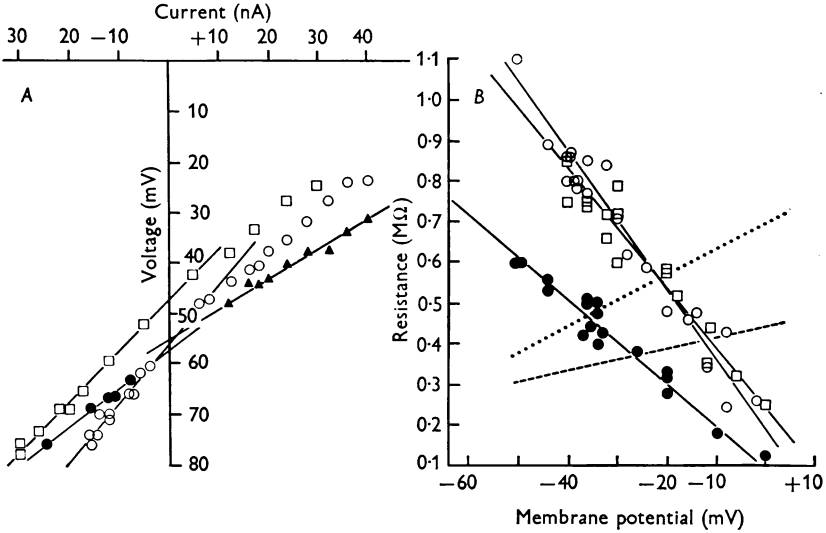

Fig. 8. Resistance measurements in motoneurone already illustrated in Fig. 5.

A, voltage–current lines drawn by eye through values obtained with 10 msec pulses. Open circles: initial control; filled circles: during Ca^{2+} injection by current of 25 nA; squares: control 60 sec after end of injection; triangles: initial sequence recorded immediately after antidromic spikes.

B, resistance estimated with hyperpolarizing current pulses (10–28 nA) at different levels of membrane potential, mainly during depolarization with various *steady* currents (0–60 nA). All values are corrected for outside controls. Open circles: first control (regression line $Y = 0{\cdot}19 - 0{\cdot}017\ X$ ($\pm 0{\cdot}00082$)); filled circles: during release of Ca, as in *A* ($Y = 0{\cdot}08 - 0{\cdot}011\ X$ ($\pm 0{\cdot}00056$)); squares: 60 sec after end of release ($Y = 0{\cdot}26 - 0{\cdot}014\ X$ ($\pm 0{\cdot}0011$)); Y and X being in units of MΩ and mV respectively. Interrupted lines represent approximately controls recorded outside while applying steady current over similar range, taking 1 mV in abscissa as equivalent to 1 nA; actual regression lines are, for first control (below): $Y = 0{\cdot}33 + 0{\cdot}003\ X$ ($\pm 0{\cdot}00074$); and for release of Ca^{2+} (above), $Y = 0{\cdot}42 + 0{\cdot}006\ X$ ($\pm 0{\cdot}00067$), Y and X being in units of MΩ and nA respectively.

diminish as the depolarizing pulses were increased. The open symbols in Fig. 8*B* show an approximately linear fall of resistance, estimated with a constant hyperpolarizing pulse, during progressive depolarization by steady positive currents (applied through the same KCl-containing barrel, cf. Fig. 1). During the release of Ca^{2+} (filled circles), the slope of resistance

against membrane potential was very significantly reduced (from $-16{\cdot}7$ to $-10{\cdot}6$ kΩ/mV). The interrupted lines show an opposite tendency in control runs performed outside the cell, that is an increase in coupling resistance during the flow of comparable positive currents, and a marked *increase* in slope when Ca^{2+} was released (upper line). It is therefore very unlikely that the effect observed intracellularly could be an electrode artifact.

In order to by-pass tip artifacts, estimates of resistance were also obtained independently by measuring changes in the amplitude of spikes or synaptic potentials caused by positive and negative polarizing currents. The tests were repeated while injecting Ca^{2+}, and further controls were performed afterwards. As pointed out by Frank & Fuortes (1956), this technique really measures the difference between the resting resistance and the resistance at the peak of the spike or synaptic potential. An example of this type of test is given in Fig. 9. An i.p.s.p. was evoked by orthodromic stimulation, and its amplitude could be varied with pulses of current, as shown by the open circles. The filled circles were recorded while injecting Ca^{2+} (12 nA below and 35 nA above). In both cases, there was a shift towards lesser negativity (or even marked positivity, above), owing to the concurrent release of Cl^-. In addition, the voltage–current slopes became appreciably less steep, especially in the region of depolarizing currents.

Similar tests were performed using the amplitude of the cell's antidromic spike instead of the i.p.s.p., yielding a somewhat higher apparent resistance (0·74 MΩ instead of 0·60 MΩ) and somewhat larger changes during the release of Ca^{2+} (an increase by 59 instead of 42 %). Although these differences were in the expected direction – since the membrane resistance should fall to a particularly low value at the peak of the spike – the standard errors of the regression coefficients suggest that they were only doubtfully significant (cf. legend of Fig. 9).

Attempts were always made to repeat tests of resistance in the same cell, using at least one other technique; but these were completely successful in only seven cases; in most cases, the intracellular recording was insufficiently stable for a second series of tests, or the electrode did not permit further adequate release of Ca^{2+}.

Summary of resistance changes

Fifty-three neurones showed some evidence of a fall in input resistance during intracellular injections of Ca^{2+}; but quantitative estimates could be made in only seventy runs on forty-one cells. These data are summarized in Table 1, which gives the mean (%) reduction in resistance observed with different rates of injection of Ca^{2+} (grouped in four classes). The inconstant effects produced by injections of 5–8 nA are reflected in the large corre-

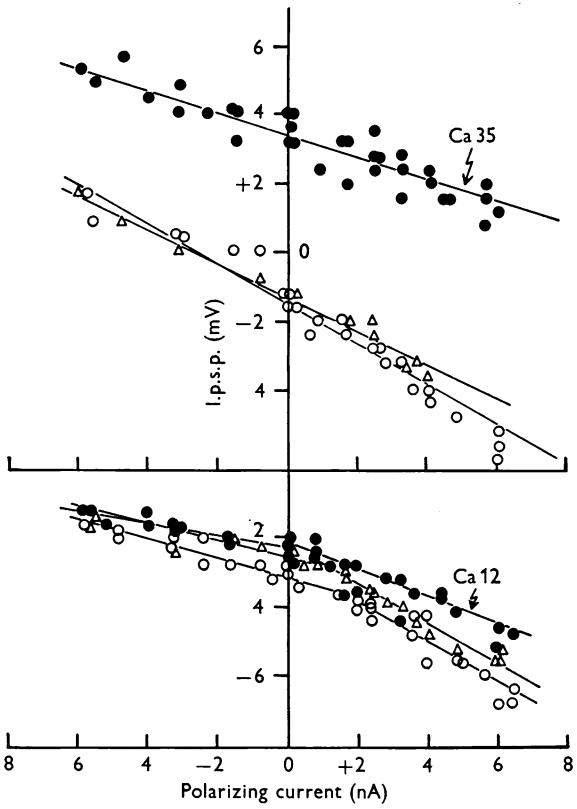

Fig. 9. Resistance measurements from changes in amplitude of orthodromic i.p.s.p. with membrane polarization. Two series are shown below and above, in each case with values before (open circles) and during Ca^{2+} injection (filled circles), as well as after end of release (triangles). Iontophoretic currents are indicated (in nA). Negative coefficients (± S.E.) for regression of potential (mV) on current (nA) were as follows. Lower series: for negative currents, before 0·27 (± 0·022), during Ca^{2+} (12 nA) release 0·20 (± 0·002), and after 0·21 (± 0·025); for positive currents, before 0·56 (± 0·026), during Ca^{2+} (12 nA) release 0·39 (± 0·032) and after 0·53 (± 0·022). Upper series: control before 0·60 (± 0·030), during Ca^{2+} (35 nA) release 0·35 (± 0·025) and after 0·50 (± 0·026).

TABLE 1. Changes in cell input resistance produced by intracellular injections of Ca^{2+} (in forty-one neurones)

No. of observations	Ca^{2+}-releasing current (nA)	Mean fall in resistance (%)	S.E. of mean
6	5–8	11·2	4·83
23	10–12	13·6	2·54
32	18–30	36·1	4·07
9	35–50	41·6	6·79

sponding standard error. It appears that the minimum rate of injection required for a consistent effect was $\approx$ 10 nA and that a maximal change was approached with injections by currents of 18–30 nA.

In Fig. 10, we have plotted the fall in resistance produced by two or more doses of Ca^{2+} in each of eleven cells. There was some suggestion of saturation at the higher rates of injection. The slopes of fall in resistance against injection currents varied from 0·33 to 4·4 % per nA, with a mean of 1·70 % per nA (S.E. 0·305, $n = 15$).

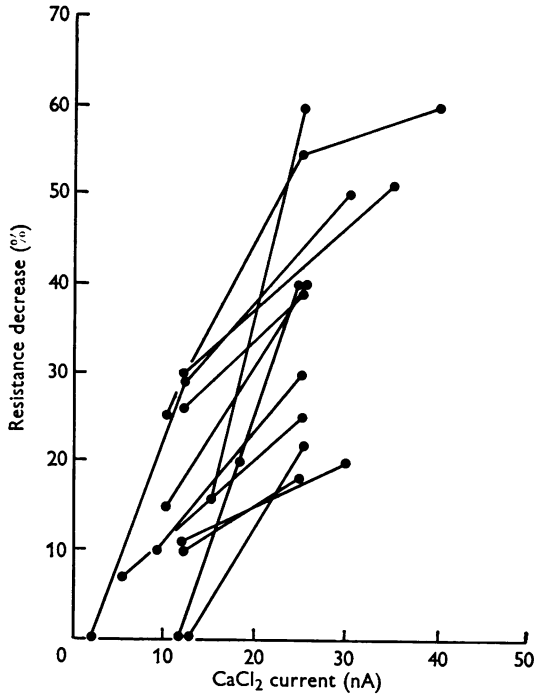

Fig. 10. Fall in resistance (% of initial value) as a function of Ca^{2+} releasing currents, observed in twelve different neurones. All points joined by lines are from a single cell.

Changes in membrane potential

These were probably the least reliable observations, owing to the variable coupling artifacts associated with injections of Ca^{2+}, which nearly always tended to make the electrode tip more positive (cf. Fig. 2). After making some allowance for these artifacts, it appears that thirty-eight injections of Ca^{2+} (into twenty-eight neurones) caused a relatively clear hyperpolarization in 53 % of cases, a depolarization in 26 % of cases, and had no clear effect in the remainder. The mean hyperpolarization was $-5{\cdot}0$ mV (S.E. 0·70) and the mean depolarization $+7{\cdot}0$ mV (S.E. 2·02). Thus the

changes in potential were more often towards increased negativity, but they were relatively small; they cannot be considered much more than suggestive, especially in view of the serious errors introduced by artifacts which could be only partly controlled.

Reversal potential for the action of Ca^{2+}

When conditions were particularly stable, and coupling artifacts slight, it was possible to obtain enough data to construct a series of voltage–current lines describing reliably the membrane properties before, during

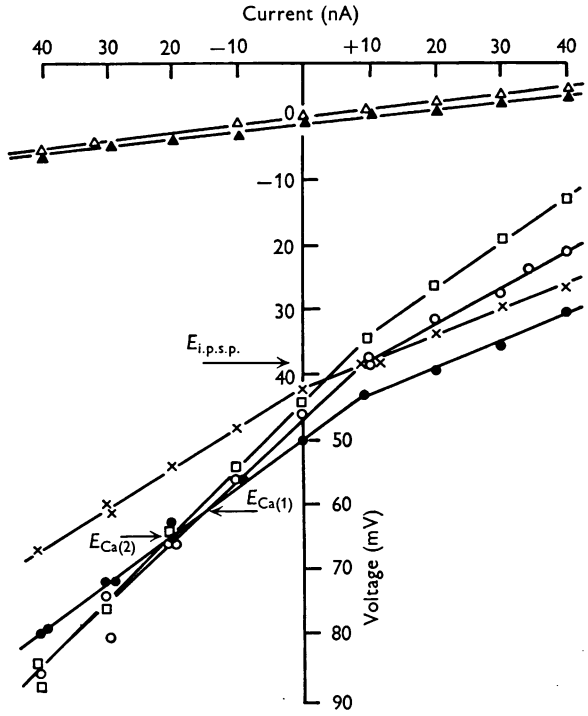

Fig. 11. Voltage–current points obtained by applying 10 msec pulses of current, in resting state (open circles), during release of Ca^{2+} (12 nA; filled circles) and 1 min later (open squares). Crosses indicate values recorded during an orthodromic i.p.s.p. Open and filled triangles show control series recorded outside cell, before and during identical release of Ca^{2+}. Arrows point to intersections of lines indicating reversal levels for i.p.s.p. and for effect of Ca^{2+}. Further data from same cell in Figs. 12–14.

and after injections of Ca^{2+}. From the points of intersection of such lines, one could estimate the reversal level for any potential change produced by Ca^{2+}.

A series of voltage–current lines obtained in one experiment are given in Figs. 11–13. Although the bridge was not quite balanced, as shown by

the slope recorded outside the cell (cf. lines at top), there was only a minimal shift in tip potential during the release of Ca^{2+} and no significant change in resistance (cf. filled and open triangles). All the values recorded inside the cell (below and also in Figs. 12 and 13) were corrected for this amount of bridge imbalance.

After an initial series of tests (not shown here, but cf. Fig. 14, which summarizes the main data obtained from this cell), a control run gave the open circles. This was followed by an injection of Ca^{2+} (12 nA), during which were recorded the values marked as filled circles. A control run was performed 30–60 sec after the end of the injection of Ca^{2+} (open squares).

Because of the consistently different slopes in the negative and positive halves of the graphs of Figs. 11–13, two straight lines were drawn (by eye) for nearly every set of points. This change of slope presumably reflects rectifying properties of the cell membrane, and not an electrode artifact, since the extracellular control points show no sign of a corresponding inflexion. Although the values of resistance were calculated from the slopes in the negative halves, qualitatively similar results are obtained from the slopes in the positive halves.

The lines drawn through these points clearly show that the injection of Ca^{2+} led to a fall in resistance (to 0·60 MΩ) from control levels of 0·85 and 0·90 MΩ) and a small hyperpolarization (− 5 mV), whose reversal potential (E_{Ca}) – at the intersection of the lines (− 61 or − 65 mV) – is some 15–20 mV more negative than the resting potential. For comparison, the current voltage points recorded during orthodromically evoked i.p.s.p.s, simultaneously with the initial control points, are shown as crosses in Fig. 11: one can see that the reversal level for the i.p.s.p. ($E_{i.p.s.p.}$) was at − 38 mV, or 8 mV more positive than the corresponding resting potential.

For the sake of convenience, the reversal levels for the changes in membrane potential induced by the intracellular injection of Ca^{2+} have been labelled E_{Ca}; but of course this is not meant to indicate any similarity to the equilibrium potential for Ca^{2+}.

As a further control, an iontophoretic current of 12 nA was made to flow in the opposite direction, from the KCl- to the $CaCl_2$-containing barrel; during this time, the points indicated by filled circles in Fig. 12 were recorded, giving a line indistinguishable from the previous control line (open circles). A final control run (open squares) also yielded a line with a practically identical slope.

By this technique it was possible to obtain twelve estimates of the reversal potential for the effect of Ca^{2+} from five different cells (cf. Fig. 8 for another example). After grouping together the values for each of the five cells, the mean difference between reversal levels and resting potentials came to − 9·6 mV, with a range of − 2·2 to − 17 mV.

Effects of Ca^{2+} on i.p.s.p.s

On several occasions, inhibitory synaptic potentials (i.p.s.p.s) were evoked by orthodromic or antidromic stimulation. Injections of Ca^{2+} were associated with a corresponding release of Cl^-, which might be expected to reduce or reverse the initially negative i.p.s.p.s. This effect has indeed already been seen in Fig. 9 (especially during the larger application of current, in the upper graph).

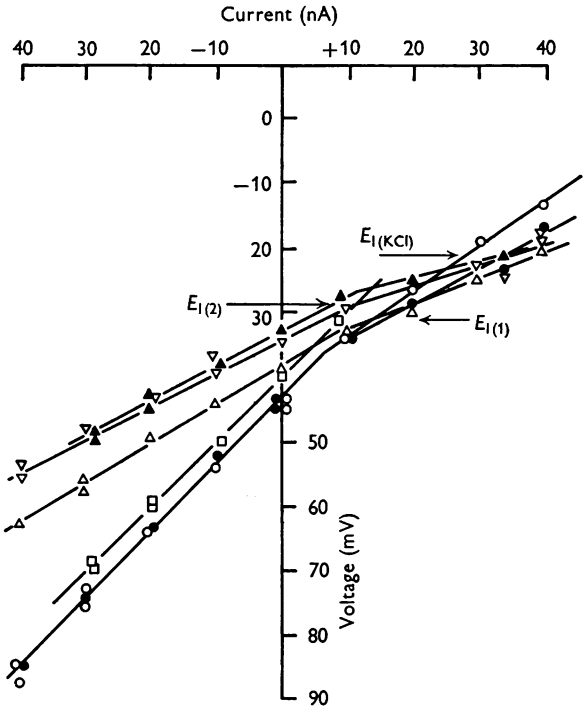

Fig. 12. Control injection of KCl (12 nA) into same cell (cf. Figs. 11, 13 and 14). Resting voltage–current points were obtained before (open circles), during (filled circles) and one min after injection (open squares). I.p.s.p. values recorded at same time are indicated by triangles: △—△ before, ▲—▲ during injection, ▽—▽ after. Arrows show corresponding i.p.s.p. reversal levels (E_I).

It is also evident that leakage of Cl^- is causing a reversal of i.p.s.p.s in the experiment already illustrated in Figs. 11 and 12. The triangles in Fig. 12 give the i.p.s.p. voltage–current points recorded almost simultaneously with the resting values. They indicate a reversible increase in i.p.s.p. positivity during the injection of KCl (confirmed by a sharply more positive i.p.s.p. reversal potential, cf. arrows) but little change in resistance.

In contrast to this, the i.p.s.p. became *less* positive and its resistance clearly reduced during a similar iontophoretic current releasing inside the cell Ca^{2+} instead of K^+. This is shown by the i.p.s.p. data in Fig. 13, which were recorded at the same time as the resting values illustrated in Fig. 11, but are shown separately for the sake of clarity: the corresponding resting slopes are indicated by dotted lines. It is evident from the intersections of

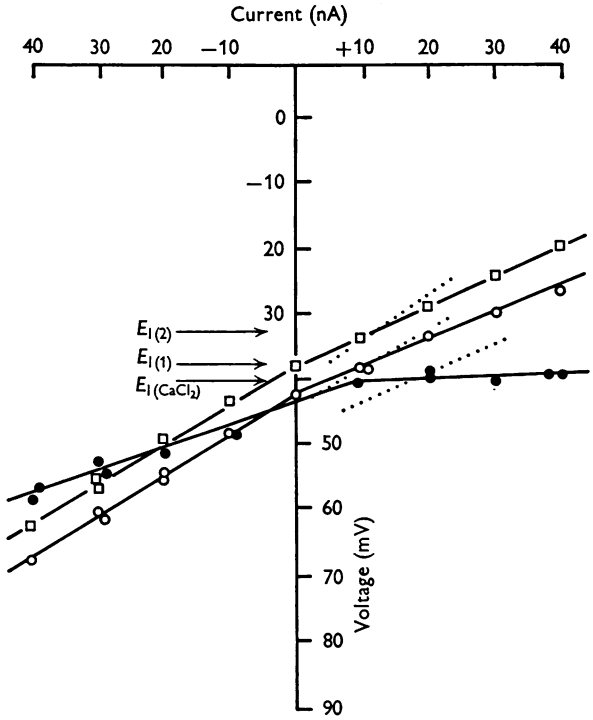

Fig. 13. Effect of $CaCl_2$ (12 nA) injection on i.p.s.p. voltage–current lines; open circles are values recorded before injection, filled circles during injection and open squares 1 min later. This series was obtained at same time as resting voltage–current lines of Fig. 11, whose slopes are shown by dotted lines to indicate i.p.s.p. reversal levels.

the i.p.s.p. and resting lines (marked by arrows) that the i.p.s.p. reversal level became more *negative* during the injection of $CaCl_2$, but its subsequent control value was 5 mV more positive than the initial level.

All the values of the i.p.s.p. peak potential, resistance and reversal potential recorded in this cell are plotted in Fig. 14. This brings out very clearly the contrast between the effects of the similar but opposite iontophoretic currents: the release of KCl did not change the resistance, but it caused a large *positive* shift in i.p.s.p. reversal level; whereas the release

of $CaCl_2$ led to a marked fall in resistance, associated with a small *negative* shift in i.p.s.p. reversal level.

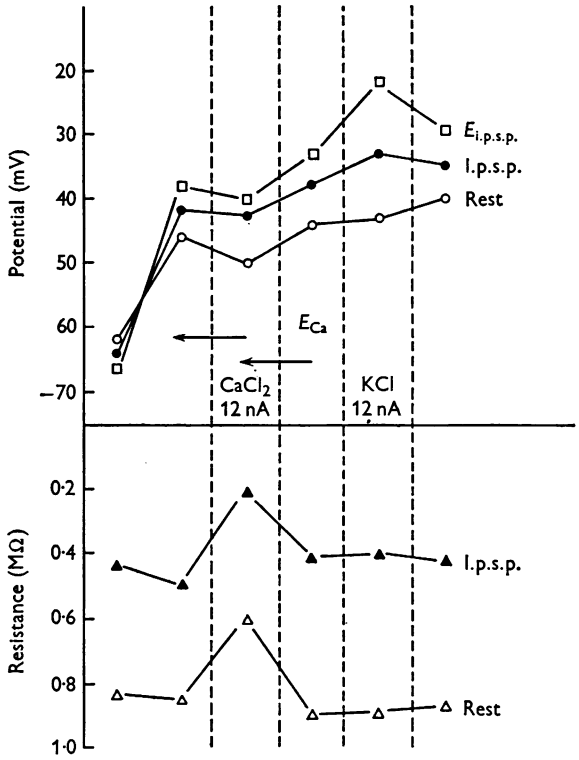

Fig. 14. Values of resting potential (open circles), potential at peak of i.p.s.p. (filled circles) and reversal level for i.p.s.p. (open squares); as well as resting resistance (open triangles) and resistance during i.p.s.p. (filled triangles), all recorded in six separate runs over a total period of approximately 30 min, during experiment illustrated in Figs. 11–13. Arrows indicate reversal level for Ca^{2+} action.

Interaction between Ca^{2+} and post-spike hyperpolarization

In five cases voltage–current points were recorded during the after-potential. The corresponding lines indicated a lower membrane resistance (cf. triangles in Fig. 8*A*). There was some difficulty in analysing the interaction between Ca^{2+} and this phenomenon, because spikes tended to disappear when the effects of Ca^{2+} were fully developed. In three cases out of five, the intracellular release of Ca^{2+} appeared to abolish or greatly diminish (by more than half) any further reduction in resistance after the spikes. The reversal potential for the after-hyperpolarization could be compared with that for the Ca^{2+} effect only in two cells: in both cases they did not differ by > 2 mV, and there was no significant change during the release of Ca^{2+}.

DISCUSSION

In spite of the technical difficulties, which restricted the number of precise observations, certain features were recorded consistently: intracellular injections of Ca^{2+} tended to lower the motoneurones' excitability and input resistance, and, less regularly, made its membrane potential more negative. These effects were rapid in onset and apparently reversible.

Amounts of Ca^{2+} injected and changes in intracellular concentration

Taking the simplest view, that is assuming no loss by absorption within the cell or by diffusion or transport out of the cell, the mean intracellular concentration would change as a simple function of the rate of injection and the cell volume (v)

$$\frac{\delta c}{\delta t} = \frac{In}{Fzv}, \tag{1}$$

where I is the iontophoretic current, n the transport number for the ion injected, z its valency, and F Faraday's constant.

According to the data of Table 1, the minimum current needed for a clear effect was $\approx$ 10 nA. If we assume a transport number of 0·40 for Ca^{2+} (Kortüm & Bockris, 1951) this current gives a Ca^{2+} flux (F_0) of 20 f-mole/sec (1 f-mole = 10^{-15} mole).

By direct observation of cells labelled with tritiated glycine, Lux, Schubert & Kreutzberg (1970) found that spinal motoneurones with input resistance of < 1 MΩ have a somatic surface area of $\approx 100 \times 10^{-6}$ cm². If one approximates the soma to a sphere of similar surface area, its volume would be (surface area)$^{\frac{3}{2}}/6\pi^{\frac{1}{2}}$ or $\approx$ 100 pl.

Since most of the cells we studied had a low resistance (< 1 MΩ), presumably because the relatively large-tipped electrodes could record satisfactorily only from the larger cells, they would have a comparable volume ($\approx$ 100 pl.) and this rate of injection would increase the total internal Ca^{2+} by $\approx$ 20 f-mole/100 pl./sec or $\approx$ 0·2 mM/sec. If maintained for 1 min, even a small iontophoretic current could raise the concentration to $>$ 10 mM. The normal free Ca^{2+} concentration inside the cell is probably $\ll$ 1 mM (Keynes & Lewis, 1956; Hodgkin & Keynes, 1957; Portzehl, Caldwell & Rüegg, 1964; Baker, Hodgkin & Ridgway, 1971): this rate of increase is therefore very high, and should cause an irreversible block rather than just detectable effects (cf. Hodgkin & Keynes, 1956; Tasaki, 1968).

A possible explanation for this discrepancy is that the volume (v) has been grossly underestimated; this could happen if our measurements of cell resistance were seriously in error. However, the effects of injections of KCl on the i.p.s.p. reversal level (E_I) suggest that this was not the case.

If the i.p.s.p. is caused by increases in both g_{Cl} and g_K (Eccles, 1964),

$$E_I = T_{Cl}E_{Cl} + (1 - T_{Cl})E_K, \tag{2}$$

where T_{Cl} is the fraction of total current carried by Cl^-.

One can assume that T_{Cl} and E_K remain constant for relatively small changes in internal Cl^- concentration $([Cl]_i)$; therefore (cf. Hodgkin & Horowicz, 1959), at 37°

$$\frac{\delta E_I}{\delta [Cl]_i} = \frac{60 T_{Cl}}{[Cl]_i}. \tag{3}$$

Ignoring any removal of Cl^- from the cell, by combining eqns. (1) and (3) we can use the observed rate of change of E_I to estimate the cell volume

$$v = \frac{In60T_{Cl}}{Fz[Cl]_i(\delta E_I/\delta t)}. \tag{4}$$

Let us consider, as a specific example, the motoneurone illustrated in Figs. 11–14: E_I changed from -33 to -22 mV as a result of injecting KCl with a current of 12 nA for about 40 sec; this gives a mean $\delta E_I/\delta t$ of 0·28 mV/sec.

We can now calculate a range of values of v consistent with the observed $\delta E_I/\delta t$, since $[Cl]_i$ can be obtained from eqn. (2) and the Nernst equation ($[Cl]_o$ is taken to be 148 mM (cf. Ames, Higashi & Nesbett, 1965)). According to Eccles (1964), T_{Cl} is $\leqslant \frac{2}{3}$, but the more recent observations of Lux (1971) suggest a value closer to 1·0. If $T_{Cl} = 1{\cdot}0$, $[Cl]_i$ is 42 mM, and, from eqn. (4), $v = 300$ pl. But if $T_{Cl} < 1{\cdot}0$, $[Cl]_i$ is a function of both T_{Cl} and E_K; we can assume that E_K lies between -100 mV (its normal value) and -60 mV (more in keeping with the resting potential of -45 mV recorded at this time; cf. Fig. 14); therefore, taking $T_{Cl} = 0{\cdot}67$ (its probable lower limit), we have two possible values of $[Cl]_i$, 148 and 100 mM respectively, and two corresponding values of v, 59 and 87 pl. The fair agreement between these calculated dimensions and those predicted from the data of Lux *et al.* (1970) suggests that our estimates of resistance could not be very much in error.

Clearly, it is the simple assumption, that all the Ca^{2+} injected remains free, which is most questionable. As in serum (Moore, 1969) there must be some binding to protein and smaller anions; but in addition, Ca^{2+} is probably removed by uptake into mitochondria (Vasington & Murphy, 1962). This aspect of mitochondrial function has been studied extensively (cf. recent reviews by Lehninger, Carafoli & Rossi, 1967; Lehninger, 1970; Carafoli & Rossi, 1971). Mitochondria from all tissues (including brain) have been shown to absorb Ca^{2+} against a high concentration gradient. At 25° C, during 'limited loading', Ca^{2+} is absorbed at a rate of 1–6 n-mole/mg mitochondrial protein per second (E. Carafoli, personal communication), the total capacity being some 150 n-mole Ca^{2+}/mg. Since the uptake process has a K_M of 10^{-6} M, the cytoplasmic concentration would be maintained near this level, as is found under normal conditions (Baker *et al.* 1971). In the presence of permeant anions, a further 'massive' loading becomes possible, which greatly increases the total uptake.

Since mitochondria are evenly scattered throughout the cytoplasm (cf. Peters, Palay & Webster, 1970), an injection of Ca^{2+} at any point must

initiate Ca^{2+} uptake which will prevent a significant rise in Ca^{2+} concentration beyond a certain distance from the site of injection.

If there is a constant flux of Ca^{2+} from the tip of the micropipette, this can be assumed to act as a continuous point source in an infinite homogeneous medium where Ca^{2+} moves freely (with a diffusion coefficient D), but is absorbed at a constant rate per unit volume (a); the change in concentration (c) with time (t) and at a radial distance (r) from the tip is therefore (cf. Hill, 1928; Carslaw & Jaeger, 1959):

$$\frac{\delta c}{\delta t}+a = D\left(\frac{\delta^2 c}{\delta r^2}+\frac{2}{r}\frac{\delta c}{\delta r}\right). \tag{5}$$

A steady state is soon reached, when $\delta c/\delta t = 0$, and now

$$\frac{\delta^2 c}{\delta r^2}+\frac{2}{r}\frac{\delta c}{\delta r} = \frac{a}{D}. \tag{6}$$

By integration of (6), we obtain

$$\frac{\delta c}{\delta r} = \frac{ar}{3D}+\frac{K_1}{r^2} \tag{7}$$

and

$$c = \frac{ar^2}{6D}-\frac{K_1}{r}+K_2, \tag{8}$$

where K_1 and K_2 are constants of integration. By definition, the total flux of Ca^{2+} at any distance r is

$$F = -4\pi r^2 D\frac{\mathrm{d}c}{\mathrm{d}r}, \tag{9}$$

$$F = -(4/3\pi ar^3+4\pi DK_1). \tag{10}$$

At $r = 0$, the flux is equal to the flux from the micropipette (F_0), hence

$$K_1 = -\frac{F_0}{4\pi D}. \tag{11}$$

At a certain distance r_0, there is no increase in concentration, and therefore $\mathrm{d}c/\mathrm{d}r = 0$. Substituting in (7),

$$\frac{ar_0}{3D}-\frac{F_0}{4\pi Dr_0^2} = 0 \tag{12}$$

from which

$$r_0 = \sqrt[3]{\left(\frac{3F_0}{4\pi a}\right)}. \tag{13}$$

By means of eqn. (13) one can find out whether the amounts of Ca^{2+} which had to be injected to evoke a clear effect are consistent with the

most probable dimensions of the motoneurones and the likely rates of Ca^{2+} uptake by mitochondria.

The simplest hypothesis is that Ca^{2+} depresses excitability by a direct action on the cell membrane (or its immediate vicinity).

Ca^{2+} liquifies squid axoplasm (Hodgkin & Katz, 1949; Hodgkin & Keynes, 1956), but it is not obvious why this should reduce excitability. Most of the axoplasm can be replaced by liquid perfusates without affecting impulse conduction (Baker *et al.* 1962; Tasaki, Watanabe & Takenaka, 1962), though the addition of Ca^{2+} rapidly abolishes excitability (Baker *et al.* 1962; Tasaki, 1968). Since ionic mobility is not restricted in aqueous gels (Stiles & Adair, 1921), a change in axoplasmic consistency is also unlikely to alter seriously the resistance seen by an intracellular electrode.

One can postulate that the critical conditions arise when the rate of injection is just sufficient to produce a significant increase in free Ca^{2+} at the inner aspect of the cell membrane. In the region of the soma, a motoneurone of 0·85–0·90 MΩ resistance has a radius of $\approx$ 25 μm (cf. Lux *et al.* 1970). From a minimum required rate of injection of 20 f-mole/sec (see above), the rate of Ca^{2+} uptake that would just prevent any change in concentration at a distance of 25 μm is given by (cf. eqn. (13))

$$a = \frac{3F_0}{4\pi r_0^3} \qquad (14)$$

$$= 320 \text{ n-mole/ml. sec.}$$

Since mammalian neurones have a protein content of 100–200 mg/ml. (Lowry, Roberts, Leiner, Wu, Farr & Albers, 1954; Hydén, 1962; Levi, 1969), of which 15–25 % is probably in mitochondria (Lehninger, 1964; Abood, 1969), this rate of uptake is equivalent to 6–20 n-mole/mg mitochondrial protein per sec. While this is somewhat higher than previously observed limited loading at 25° C, it is not excessive for the higher temperature, especially if enough permeant anions are present to allow some massive loading. No doubt, the removal of Ca^{2+} from the critical region near the membrane must be accelerated by pumping out of the cell (Baker *et al.* 1971).

The result of these approximate calculations are thus consistent with the possibility that most of the Ca^{2+} injected during the experiments was removed by uptake into mitochondria, and that the changes in excitability were produced by only relatively small amounts of free Ca^{2+} near the membrane. This scheme may help to explain why the effects observed were very rapid and mostly reversible.

Change in membrane permeability induced by injections of Ca^{2+}

A sharp fall in membrane resistance, associated with only small changes in membrane potential – hyperpolarization predominating – could be

accounted for by an increase in mainly K^+ or Cl^- conductance (g_K or g_{Cl}). But a rise in g_{Cl} was probably not a significant factor, since the reversal level for the action of Ca^{2+} was much more negative than the reversal potentials of i.p.s.p.s (cf. Figs. 11 and 14). The contrast between the effects of injections of KCl and $CaCl_2$ was very revealing; KCl accentuated the trend towards a more positive $E_{i.p.s.p.}$ whereas $CaCl_2$ reversed this temporarily, in spite of the concomitant increase in internal Cl^-. At the same time, there was a further drop in resistance as the Ca^{2+}-induced conductance change was *added* to the high i.p.s.p. conductance. These facts are strong evidence that i.p.s.p. and Ca^{2+} operate through quite distinct ionic mechanisms; and so the main action of Ca^{2+} must be to increase g_K. This conclusion is supported by the similarity of reversal levels for the action of Ca^{2+} and for the post-spike hyperpolarization, although this evidence was too fragmentary to be more than suggestive.

These results thus agree with Meech & Strumwasser's (1970) observations that intracellular injections of Ca^{2+} raise g_K in *Aplysia* neurones. But in addition to this principal action, Ca^{2+} may also cause some increase in g_{Na}, since the resting potential in our experiments was often relatively little changed, or even became a little more positive (coupling artifacts probably exaggerated this effect). In this respect, the motoneurones may behave like red blood cells, in which a rise in free Ca^{2+} level increases first the K^+ permeability and then the Na^+ permeability (Whittam, 1968; Romero & Whittam, 1971).

Although the large increase in membrane conductance, with a ratio of g_K/g_{Na} remaining close to, or exceeding its value in the resting state, seems sufficient to explain the fall in excitability, we have not excluded the possibility that internal Ca^{2+} may increase Na^+ inactivation.

Significance of observations

The similar effects produced by removal of external Ca^{2+} (Frankenhaeuser & Hodgkin, 1957) and by intracellular injections of Ca^{2+}, in both cases, there is a sharp loss of membrane resistance, caused by a rise in g_K, strongly suggest that a steep inward gradient of Ca^{2+} is essential for the normal membrane characteristics, particularly for the low conductivity. A striking illustration of this is the demonstration that intercellular coupling is abolished by an increase in free Ca^{2+} on either side of the cell membrane (Loewenstein, 1966; Oliveira-Castro & Loewenstein, 1971).

It seems not too far-fetched to suppose that the entry of external Ca^{2+} during the first phase of the action potential (Hodgkin & Keynes, 1957; Baker *et al.* 1971; Yamagashi & Grundfest, 1971) may be sufficient to reduce the Ca^{2+} gradient within the membrane and thus trigger the phase of delayed rectification, during which g_K is raised to a high level. The

increase in permeability may even allow some other cytoplasmic contents to pass through the membrane, such as chemical transmitters. This mechanism would explain the marked dependence of transmitter release on external Ca^{2+} (Katz, 1969; Rubin, 1970).

A release of Ca^{2+} from intraneuronal stores may have some protective value. If metabolic activity is reduced, whether owing to an inadequate supply of O_2 or substrate, or poisoning by specific inhibitors, the resulting slowing-down of Na-K pumping should lead to depolarization, which would accelerate firing and therefore increase the metabolic requirements of the neurone and perhaps of other parts of the organism. But if slower mitochondrial activity allows the internal free Ca^{2+} level to rise, neuronal firing would be damped down. This may well be the mechanism of the reversible hyperpolarization and fall in excitability that are observed in the cerebral cortex during hypoxia (Glötzner, 1967; Godfraind *et al.* 1971).

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